

# RNA trafficking and stabilization elements associate with multiple brain proteins

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## Summary

Two of the best understood somatic cell mRNA cytoplasmic trafficking elements are those governing localization of  $\beta$ -actin and myelin basic protein mRNAs. These cis-acting elements bind the trans-acting factors fibroblast ZBP-1 and hnRNP A2, respectively. It is not known whether these elements fulfil other roles in mRNA metabolism. To address this question we have used Edman sequencing and western blotting to identify six rat brain proteins that bind the  $\beta$ -actin element (zipcode). All are known RNA-binding proteins and differ from ZBP-1. Comparison with proteins that bind the hnRNP A2 and AU-rich response elements, A2RE/A2RE11 and AURE, showed that AURE and zipcode bind a similar set of proteins that does not overlap with those that bind A2RE11. The zipcode-binding protein, KSRP, and hnRNP A2 were selected for further study and

were shown by confocal immunofluorescence microscopy to have similar distributions in the central nervous system, but they were found in largely separate locations in cell nuclei. In the cytoplasm of cultured oligodendrocytes they were segregated into separate populations of cytoplasmic granules. We conclude that not only may there be families of trans-acting factors for the same cis-acting element, which are presumably required at different stages of mRNA processing and metabolism, but independent factors may also target different and multiple RNAs in the same cell.

Key words: RNA-protein interactions, Zipcode, RNA trafficking, Protein sequencing, Confocal microscopy

## Introduction

Localization of cellular proteins is accomplished by diverse pathways, including the trafficking and localised translation of mRNA. Selective trafficking of RNA involves the recognition of cis-acting segments, commonly in the 3' UTR, by trans-acting proteins that bind indirectly to cytoskeletal elements (Singer, 1993). RNA localization in oocytes has been studied intensively (e.g. Hazelrigg, 1998; Nakielný et al., 1997; St Johnston, 1995). By contrast, only a small number of cis-acting sequences that appear to act this way in somatic cells have so far been identified.

A general pathway for mRNA trafficking in somatic cells has been proposed, in which mRNA molecules are packaged into granules in the nucleus, then exported to the cytoplasm where they attach to microtubules or microfilaments and are transported to their destination before being translated (Barbarese et al., 1999). Two of the best-described systems are those mediating localization of the mRNAs encoding  $\beta$ -actin (Bassell et al., 1999; Kislauskis et al., 1994; Oleynikov and Singer, 1998) and myelin basic protein (MBP) (Ainger et al., 1997; Ainger et al., 1993; Carson et al., 1997). Both mRNAs have small cis-acting segments: the 54-nucleotide zipcode in the chicken  $\beta$ -actin mRNA (Ross et al., 1997) and the 11-nucleotide hnRNP A2 response element (A2RE11) in the MBP mRNA (Munro et al., 1999). These RNA segments appear to operate in more than one cell type: zipcode-dependent

transport has been demonstrated in fibroblasts (Kislauskis et al., 1994; Ross et al., 1997), where it is actin-dependent, and in neurons (Bassell et al., 1998; Zhang et al., 1999), where it is microtubule dependent. Likewise, A2RE-dependent transport occurs in oligodendrocytes (Hoek et al., 1998; Munro et al., 1999) and neurons (J. Shan et al., personal communication). An important unanswered question is whether these mRNAs in different cell types use the same, or different, trans-acting factors and trafficking pathways.

We show here that the  $\beta$ -actin zipcode recognizes a set of at least ten rat brain polypeptides from rat brain. We have identified six of the most prominent proteins by Edman sequencing and western blotting as rat homologues of KH-type splicing regulatory protein (KSRP), far-upstream element binding protein (FBP), HuC and heterogeneous nuclear ribonucleoproteins (hnRNP) E1, E2 and L. All of these proteins possess established RNA-binding motifs. The RNA-sequence-specific binding of a group of brain proteins suggests that there are varied and complex interactions that govern the post-transcriptional actions and fate of mRNAs, which may involve segments such as the zipcode, perhaps in combination with other cis-acting sequences.

We further show that zipcode and the AU-rich response element (AURE), the latter a cis-acting element that influences mRNA stability by recruiting exosomes (Mukherjee et al., 2002), bind a similar set of proteins. This set does not overlap

**Table 1. Oligonucleotides used for affinity purification of RNA-binding proteins**

Oligonucleotide	Sequence
5' zipcode	GCG GAC UGU UAC UGA GCU GCG UUU UAC ACC CUU
3' zipcode	CUU UGA CAA AAC CUA ACU UGC
A2RE	GCC AAG GAG CCA GAG AGC AUG
A2RE11	GCC AAG GAG CC
AURE	GUU UAU AAU UUU UUU AUU ACU G
NS1	CAA GCA CCG AAC CCG CAA CUG

All have biotinylated U at the 3' end. The sequences for rat zipcode, A2RE and AURE are from Schedlich et al., 1997, Ainger et al., 1997 and Munro et al., 1999, and Hamilton et al., 1999 and Tsukamoto et al., 1996, respectively.

the A2RE11-binding proteins. Our results suggest that families of trans-acting factors may recognize the same cis-acting element and participate in different steps of mRNA processing and metabolism. These trans-acting factors may also target different and multiple RNAs in the same cell. Finally, we show that although KSRP and hnRNP A2 are both widely distributed in the brain they differ in nuclear distribution and are segregated into different granules in the cytoplasm of oligodendrocytes.

## Materials and Methods

### Isolation of RNA-binding proteins

RNA-binding proteins from the brains of 21-day-old rats were isolated on superparamagnetic particles bearing the non-covalently attached oligonucleotides in Table 1, as previously described (Hoek et al., 1998). Excess biotin-labelled oligoribonucleotide (Oligos etc., Wilsonville OR) was incubated with 50 µl streptavidin-coated particles (Roche Molecular Biochemicals, Mannheim, Germany) and unbound oligonucleotide washed off. The particles were then incubated, in the presence of 10 g/l heparin (to minimize non-specific interactions), with 5 mg of brain protein for 30 minutes at 4°C. After rinsing off unbound proteins, bound proteins were eluted with 30 µl of 0.1% SDS/1 mM DTT at 25 or 65°C for 10 minutes. These proteins were separated on 12% polyacrylamide gels (Gradipore Ltd, Sydney, Australia) and stained with Coomassie brilliant blue. Except where noted otherwise, the data shown below were obtained with A2RE11, but equivalent results were obtained using A2RE.

### Protein sequencing

Proteins isolated from eight individual magnetic particle experiments, each with 50 µl of magnetic particles bearing 5' zipcode were combined, partially concentrated by vacuum centrifugation, and run in a single well of a 12% SDS/polyacrylamide gel before staining with Coomassie brilliant blue. Bands were excised from the gel and digested with trypsin as follows. The gel slices were washed in 50% CH<sub>3</sub>CN for 5 minutes, 50% CH<sub>3</sub>CN/50 mM NH<sub>4</sub>HCO<sub>3</sub> for 30 minutes and 50% CH<sub>3</sub>CN/10 mM NH<sub>4</sub>HCO<sub>3</sub> for 30 minutes and then vacuum dried. TPCK-treated trypsin (0.1 µg Promega, Sydney, Australia) in 15 µl of 10 mM NH<sub>4</sub>HCO<sub>3</sub> was added to the dried gel, which was then left at 37°C overnight. Peptides were extracted using two 1 hour incubations in 200 µl of 60% CH<sub>3</sub>CN in 0.1% TFA. Tryptic fragments were dried by vacuum centrifugation and redissolved in 20 µl of 10% CH<sub>3</sub>CN/0.1% TFA before separation on a 2.1 mm×50 mm C18 reverse-phase HPLC column (Vydac, Hesperia, CA). Optimal separation was achieved using a 10–40% acetonitrile gradient in 0.1% TFA over 60 minutes at a flow rate of 30 µl/minute. Peptides from HPLC peaks that appeared well resolved were selected for Edman microsequencing on an Applied Biosystems (Foster City, CA) Procise cLC sequencer. The levels of the tryptic peptides sequenced were generally below 1 pmol, and several were sequenced at the 200–250 femtomol level.

### Western blot analysis

Proteins were separated on 12% SDS/polyacrylamide gels before electrophoretic transfer onto PVDF (Millipore, Sydney, Australia) and incubation with primary antibody. The secondary antibodies were 1 in 1000 dilutions of either goat anti-rabbit or rabbit anti-chicken antibodies conjugated to alkaline phosphatase (Sigma). Phosphatase activity was visualized using a 4-nitro blue tetrazolium/5-bromo-4-chloro-3-indoyl-phosphate substrate (NBT). The method of Stenoien (Stenoien and Brady, 1997) was used to stain for the kinesin heavy chain, but the kinesin antibody was detected with a 1 in 12,000 dilution of rabbit anti-chicken antibody conjugated to horseradish peroxidase (Sigma) and visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, UK).

### Cell culture

Brains were excised from newborn Wistar rats and homogenized in DMEM containing 10% foetal bovine serum by triturating repeatedly with a 10 ml syringe. The homogenate was allowed to settle, and the supernatant was removed and placed in 75 cm<sup>2</sup> tissue culture flasks. After 10 days the cultures were vigorously shaken and the medium was removed to generate secondary cultures on poly-L-lysine coated coverslips. Secondary cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) before processing for immunostaining.

### Immunostaining

15-day-old Wistar rats were perfused intracardially with PBS, then 4% paraformaldehyde in PBS. Brains were fixed overnight, cryoprotected in 0.5 M sucrose in PBS, frozen, then sectioned in a cryotome to generate 30 µm thick coronal sections. Sections were rinsed three times for 5 minutes in PBS containing 0.0001% Triton X100, then microwaved twice until boiling in 0.01 M citrate buffer, pH 6.0. They were then incubated in 10% Triton X-100 PBS for 30 minutes, then in 3% normal goat serum containing 1% hydrogen peroxide for 30 minutes. After rinsing with PBS, sections were incubated in primary antibodies overnight at 4°C (Table 3.1), in biotinylated secondary antibodies for 1 hour at room temperature, and then in a 1:1000 dilution of ABC solution (Avidin Biotin Complex Elite Kit, Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Peroxidase activity was detected with DAB reaction mix (Sigma), following the manufacturer's instructions, before mounting sections in glycerol.

### Fluorescence immunostaining

The procedure for fluorescence immunostaining of tissue is as described above, until the incubation with the secondary antibody. Sections were incubated either with FITC- or TRITC-conjugated secondary antibodies or with biotinylated secondary antibodies for 2 hours at room temperature. Sections incubated with biotinylated secondaries were then incubated with FITC- or Texas Red (TR)-conjugated avidin for 1 hour at room temperature. Cultured cells were

incubated in 0.1% Triton X-100 for 10 minutes, 0.2% fish skin gelatin for 15 minutes, primary antibody for 30 minutes and secondary antibody for 30 minutes. Tissue sections and cultured cells were mounted in Vectashield containing 4'-6-diamidino-2-phenylindole (DAPI, Molecular Probes Inc, Portland, OR). Tissues and cells were imaged with a Zeiss Axiophot 2 fluorescence microscope equipped with a Variocam camera (Carl Zeiss, Oberkochen, Germany), and  $\times 40$  (0.75 NA),  $\times 63$  (1.4 NA) and  $\times 100$  (1.3 NA) lenses. Confocal images were produced with Leica TCS-NT (Leica, Inc., Deerfield, IL) or BioRad MRC 600 (BioRad, CA) confocal microscopes equipped with  $\times 63$  (1.4 NA) lenses. Multiple Z-plane images were merged, where required, using NIH Image v1.62 (National Institutes of Health, Washington, DC).

### Antibodies

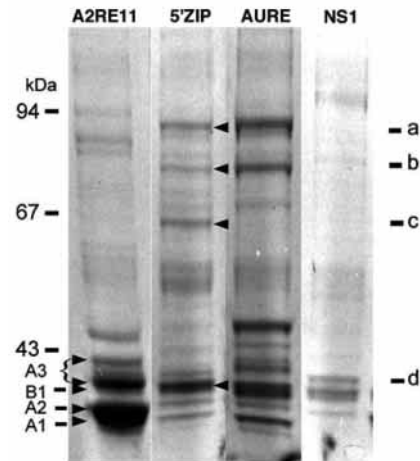
Rabbit anti-ZBP-1 antibody (Ross et al., 1997), used at a 1:100 dilution, was generously supplied by R. Singer (Albert Einstein College of Medicine, NY), chicken anti-CRD-BP antibody (Leeds et al., 1997) (1:3000) by J. Ross (McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, WI), 4606 rabbit anti-FBP antibody (1:1000) by David Levens (Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland), chicken anti-kinesin heavy chain antibody (KHC 555-772) (1:5000) by R. Diefenbach (Westmead Institute of Health Research, Westmead Hospital, Sydney, Australia) and both rabbit KSRP antibodies, one (which stains nucleoli; DBKS) raised against a C-terminal peptide and the other (C2742) against residues 172-711 (Markovtsov et al., 2000; Min et al., 1997) (1:1000) by D. Black (UCLA, CA). The specificities of our antibodies to peptides from hnRNPs A1, A2/B1, B1 and A3 have been verified previously (Ma et al., 2002) (T. P. Munro, A2RE-mediated RNA transport, PhD thesis, University of Queensland, 2002).

Other primary antibodies included mouse anti-adenomatous polyposis coli protein (CC1 antibody; Oncogene Research Products, San Diego, CA) and mouse anti-CNP antibody (Sigma). Biotin-labeled goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), goat anti-rabbit TRITC (Sigma), goat anti-rabbit FITC (Sigma), goat anti-mouse FITC (Jackson), goat anti-mouse TRITC (Sigma), mouse anti-chicken FITC (Sigma) and Texas red avidin (Vector Laboratories) were used as secondary antibodies.

### Results

#### Multiple rat brain proteins recognize the $\beta$ -actin zipcode and AURE

One of the proteins involved in transport of  $\beta$ -actin mRNA in fibroblasts has been identified as ZBP-1 (Ross et al., 1997). We sought to identify other proteins that play roles in aspects of the processing and cytoplasmic metabolism of RNAs bearing the zipcode or AURE elements. Rat brain proteins that bind to the A2RE11, 5' zipcode, AURE, and an oligoribonucleotide with the same nucleotide composition as A2RE but scrambled nucleotide sequence (NS1) were isolated in pull-down experiments using magnetic particles bearing these oligoribonucleotides (Hoek et al., 1998). More than ten zipcode- and AURE-binding polypeptides that did not bind NS1 or the A2RE11 were observed on SDS-polyacrylamide gel electrophoresis (Fig. 1). The majority of the 5'-zipcode-binding bands correlated with bands on the AURE track, but with some changes in relative intensity. The pattern of proteins binding the zipcode and AURE differed markedly from those binding the A2RE11. The prominent proteins in the A2RE11 lane (arrowheads at left in Fig. 1) are the 36 kDa hnRNP A2 (Hoek et al., 1998; Munro et al., 1999) and the two weaker,



**Fig. 1.** A2RE11, zipcode and AURE bind different groups of proteins. Coomassie-blue-stained SDS/polyacrylamide gel showing rat brain proteins that bound to magnetic particles bearing immobilized MBP mRNA trafficking sequence (A2RE11),  $\beta$ -actin mRNA 5' zipcode (5'ZIP), the AU-rich response element (AURE) or a non-specific RNA sequence (NS1). This figure was created from an image of a single gel by deletion of every second track, which contained irrelevant samples. The positions of standard proteins are shown on the left, with molecular masses in kDa.

unresolved doublets immediately above hnRNP A2 contain splice variants of hnRNP A3 (Ma et al., 2002). These proteins are involved in cytoplasmic RNA trafficking. The minor hnRNP A1 band is not resolved from the intense hnRNP A2 band, and the weak hnRNP B1 band travels just below the hnRNP A3 bands (arrowheads).

#### Identification of zipcode-binding brain proteins

Microsequencing of tryptic peptides generated by in-gel digestion after separation on SDS/polyacrylamide gels was used in an attempt to identify the most prominent of the 5'-zipcode-binding proteins. Four bands (a-d in Fig. 1, arrowheads on 5' ZIP lane) yielded levels of peptide sufficient for Edman microsequencing, all in the range of 200-500 femtomol. Three peptides from an 83 kDa band (Fig. 1, band a) matched the amino-acid sequence of human KSRP (Table 2), an RNA-binding protein that contains several KH domains; a 72 kDa band (band b) matched FBP exactly over one 11 residue peptide; three peptides from the 61 kDa band (band c) had 100% sequence identity with human hnRNP L; and a 38 kDa band (band d) yielded three peptides of which two matched the sequence of mouse HuC and one matched hnRNP E. The only difference in this region between the isoforms hnRNPs E1 and E2 is at residue 107, which is Thr in hnRNP E1 and Ser in E2: both PTH derivatives were unequivocally present in the corresponding Edman cycle, suggesting that both isoforms, which differ in mass by only 1054 Da, were present in the gel band and, therefore, that both bind the zipcode. hnRNP L appeared not to bind AURE although it bound the zipcode (band c in Fig. 1). Lower molecular weight bands corresponding in position to hnRNPs E and HuC were also evident in AURE-binding proteins. No other sequences in the translated DNA databases matched as well as those identified above, even allowing for minor ambiguities or gaps in some of



Table 2. Identification of RNA-binding proteins

Sequenced band and apparent mass/kDa*	Amino-acid sequence	Identity and accession number
a (83)	G <sup>‡</sup> ETIKQLQER	KSRP (253-262; U94832)
a (83)	MILIQDGSQNTD <sup>§</sup> VDK	KSRP (267-281)
a (83)	QFKQDDGTGPEK	KSRP (357-368)
b (72)	GNEGIDVPIPR	FBP (274-284; U05040)
c (61)	V <sup>‡</sup> QAMVEFD <sup>‡</sup> SVQSA <sup>¶</sup> Q	hnRNP L (201-214; P14866)
c (61)	NV <sup>‡</sup> FKND <sup>‡</sup> QD <sup>‡</sup> TWD <sup>‡</sup> YT <sup>¶</sup> NP	hnRNP L (243-257)
c (61)	GPQYGHPPPPPPPP	hnRNP L (339-352)
d (38)	NLYVS <sup>‡</sup> GLPK	HuC (126-134; Q60900)
d (38)	ALLTH LYQSS <sup>¶</sup> AR	HuC (213-224)
d (38)	VPAT <sup>**</sup> QC <sup>¶</sup> GSLIGK	hnRNP E1 (104-115; Q15365)
		hnRNP E2 (104-115; Q15366)

\*Band labels correspond to Fig. 1. Apparent molecular mass as measured on SDS/polyacrylamide gels in kDa in parenthesis.  
†(X-X) indicates the positions of the matching residues in the published amino-acid sequence. The accession numbers are from the GenBank or SwissProt databases.  
‡The identification of the residue before the symbol is tentative.  
§The residue before the symbol was unequivocally D and thus differs from the N in this position in human KSRP.  
¶The assignment given is from the published sequence and is consistent with the Edman results, but no PTH-amino acid was detected in this cycle.  
\*\*Both PTH-threonine and PTH-serine were detected in this cycle.

the peptide sequences. Several other bands were subjected to digestion with trypsin, HPLC and Edman degradation but yielded no reliable sequences.

Western blots highlight the specificity of RNA-protein interactions

As antibodies were available for two of the identified zipcode-binding proteins, KSRP and FBP, these proteins were selected for further study. Their specific association with zipcode was verified by western blotting. Antibodies to KSRP and FBP recognized 83 kDa (calculated mass 72 kDa) and 72 kDa (calculated mass 68 kDa) proteins, respectively, eluted from immobilized 5' zipcode but not from A2RE11 or NS1 (Fig.

2A), in accordance with the results shown in Fig. 1. Conversely, antibodies to hnRNPs A1, A2/B1, B1 and A3 recognized proteins eluted from immobilized A2RE11 but none from 5' zipcode. AURE did not bind to hnRNP A2 and is thus unlikely to bind to the other hnRNP A/B proteins (data not shown). These results demonstrate the highly sequence-specific nature of these RNA-protein interactions.

Fig. 2B shows NS1-, AURE- and 5'-zipcode-binding proteins detected with anti-KSRP (C2742) and verifies that the co-migrating AURE- and 5'-zipcode-binding proteins marked 'a' in Fig. 1 are both KSRP. In the original gel, a closely spaced doublet was observed, which may represent isoforms of KSRP.

Zipcode-binding proteins from chicken and rat were also detected on western blots with an anti-CRD-BP antibody, which recognizes ZBP-1 and the homologous KSRP protein (16% amino acid identity and 42% amino acid similarity), to determine whether ZBP-1 is a rat brain zipcode-binding protein. Both 83 kDa (data not shown) and, at a much lower level, 68 kDa ZBP-1-like proteins were present in foetal brain

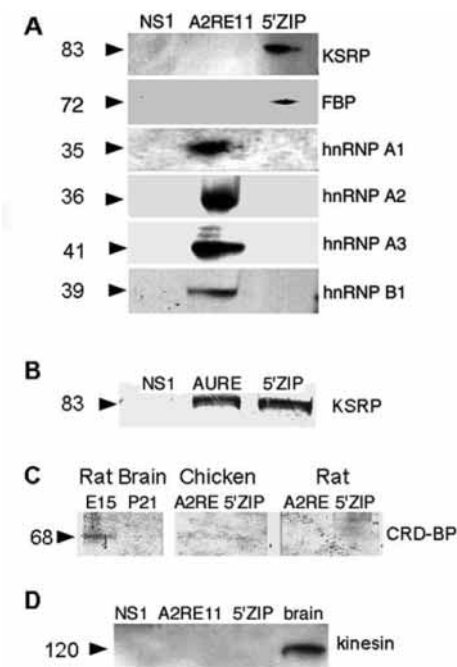


Fig. 2. Western blots confirm specificity of binding to zipcode and A2RE11. (A) 21-day-old rat brain proteins were used in pull-down experiments with magnetic particles bearing NS1, A2RE11 or 5' zipcode. The RNA-bound proteins were eluted, run on SDS/polyacrylamide gels and electroblotted. The blots were incubated with the indicated primary antibodies and protein bands detected with alkaline phosphatase-conjugated secondary antibody. The molecular masses in kDa are indicated on the left. (B) NS1-, AURE- and 5'-zipcode-binding proteins detected with anti-KSRP (C2742). (C) Western blots developed with chicken antibody to CRD-BP, which binds ZBP-1. On the left, proteins from embryonic (E15) and 21 day postnatal (P21) rat brains. No 68 kDa protein was observed in pull-down experiments with the A2RE or 5' zipcode and P21 chicken (centre) or P21 rat (right) brain protein. This blot was overdeveloped to show the weak band. (D) Western blot of rat brain proteins bound to NS1, A2RE11 and 5' zipcode detected with antibodies to conventional kinesin heavy chain. The positions of standard proteins are shown on the left, with molecular masses in kDa.

(E15, Fig. 2C), but in 21-day-old rat brain (P21) the 83 kDa protein was much less abundant and the 68 kDa band was not detected even after prolonged exposure. The 68 kDa CRD-BP-immunoreactive protein was not detected in the P21 chicken or rat brain proteins eluted from magnetic particles bearing either A2RE or 5' zipcode. ZBP-1 was, therefore, not one of the brain zipcode-binding proteins shown in Fig. 1. Only the 83 kDa protein was detected with anti-CRD-BP antibody in the chicken and rat brain proteins bound to magnetic particles bearing the zipcode, most probably reflecting the very low level of ZBP-1 in mature rat brain.

Conventional kinesin, which has been implicated in the cytoplasmic trafficking of RNAs, was evident on western blots of whole rat brain extracts but not in 5'-zipcode- or A2RE11-binding proteins (Fig. 2D). This suggests that if kinesin does bind, directly or indirectly, these RNA-binding proteins the interaction does not persist in the pull-down experiments.

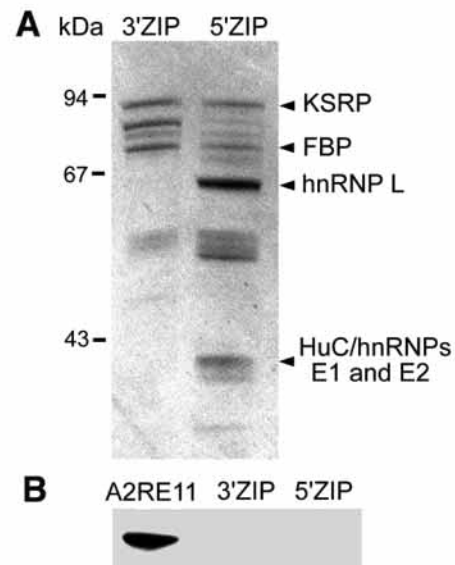
### The 3' zipcode binds a subset of the 5'-zipcode-binding proteins

Pull-down experiments were performed with rat brain proteins and the two segments of the zipcode, the 5' and 3' zipcodes, and the bound proteins analyzed on SDS/polyacrylamide gels. In this experiment, as in some others, the level of hnRNP L isolated was higher than in the experiment shown in Fig. 1. Fig. 3 shows that some, but not all, of the proteins that bound the 5' zipcode also bound the 3' zipcode. KSRP and FBP, but not hnRNP L, HuC or hnRNPs E1 and E2, bound the 3' zipcode, suggesting that within the full 51 nucleotide rat zipcode there are two sequence-specific binding sites or a site that overlaps the junction between the 5' and 3' zipcodes for the first two proteins. There is some sequence similarity between short sections of the two zipcode fragments: one possible common motif is (G/C)UUUNNNA, which is also found in AURE.

### A2RE11- and zipcode-binding proteins are co-expressed but have different distributions in brain

KSRP and FBP are widely distributed in rat brain. They were detected by DAB staining in different cell types in the white (wm) and grey (gm) matter of 15-day-old animals. From their position and morphology, most cells, including oligodendrocytes, astrocytes and cortical and hippocampal neurons, appeared to be positive for KSRP, FBP and hnRNP A2.

The distribution of KSRP and FBP in the cortex is shown in Fig. 4A,C. Intense KSRP and FBP fluorescence was found in the nuclei of cells stained for the oligodendrocyte cell body marker CC1 (Fig. 4B,D). These cells also expressed hnRNP A2 (Fig. 4E,F), suggesting that A2RE-binding proteins (hnRNP A2) and zipcode-binding proteins (KSRP-immunoreactive proteins) are co-expressed. Although KSRP and FBP were detected primarily in the nuclei by DAB staining the hnRNP A2 appeared to be present in both the nuclei and cytoplasm (Fig. 4E). However, confocal fluorescence microscopy showed that most hnRNP A2 is nuclear in oligodendrocytes (Fig. 4F). hnRNP A3, which, like hnRNP A2, may play a role in cytoplasmic trafficking of RNA (Ma et al., 2002) was also apparent in oligodendrocyte nuclei. hnRNP A1, which appears not to associate with A2RE, or to do so more weakly, was not evident, and hnRNP B1, a far less



**Fig. 3.** The 3' zipcode binds a subset of the 5'-zipcode-binding proteins isolated from rat brain protein extracts. (A) Coomassie-blue-stained SDS/polyacrylamide gel showing rat brain proteins that bound to magnetic particles bearing immobilized  $\beta$ -actin mRNA 5' zipcode (5'ZIP) or the 3' zipcode (3'ZIP). The bands identified by Edman sequencing are marked on the right. In this experiment, as in others we have performed, the level of hnRNP L was higher than in the experiment shown in Fig. 1. The positions of standard proteins are shown on the left, with molecular masses in kDa. (B) Western blotting of proteins from pull-down experiments performed with immobilized A2RE11, 5'ZIP and 3'ZIP showed that hnRNP A2 does not bind the 5' zipcode or 3' zipcode. The blot was developed with hnRNP A2 antibody as the primary antibody. Protein bands were detected with an alkaline-phosphatase-conjugated secondary antibody.

abundant isoform of hnRNP A2, was detected in only a small subset of oligodendrocytes (Fig. 4G,H).

### KSRP and hnRNP A2 have different nuclear locations in oligodendrocytes

The co-expression of KSRP-immunoreactive proteins and hnRNP A2 was also evident in cultured glial cells, where both are present predominantly in the nuclei (Fig. 5A,B). A single confocal optical section shows that KSRP-immunoreactive proteins and hnRNP A2 had a reticular distribution in the nucleoplasm and appeared to be excluded from many small circular nuclear regions. There was some overlap in the reticular staining patterns, but both proteins were concentrated in different regions. The difference in the distributions of these proteins was most striking in mitotic cells in which KSRP was widely distributed in the mitotic cell (Fig. 5D), whereas hnRNP A2 (Fig. 5E) was primarily associated with the segregated chromosomes (Fig. 5F,G).

### KSRP and hnRNP A2 are in different cytoplasmic granules

In oligodendrocyte processes, antibodies to KSRP and hnRNP A2 bound cytoplasmic granules, which resemble the mRNA



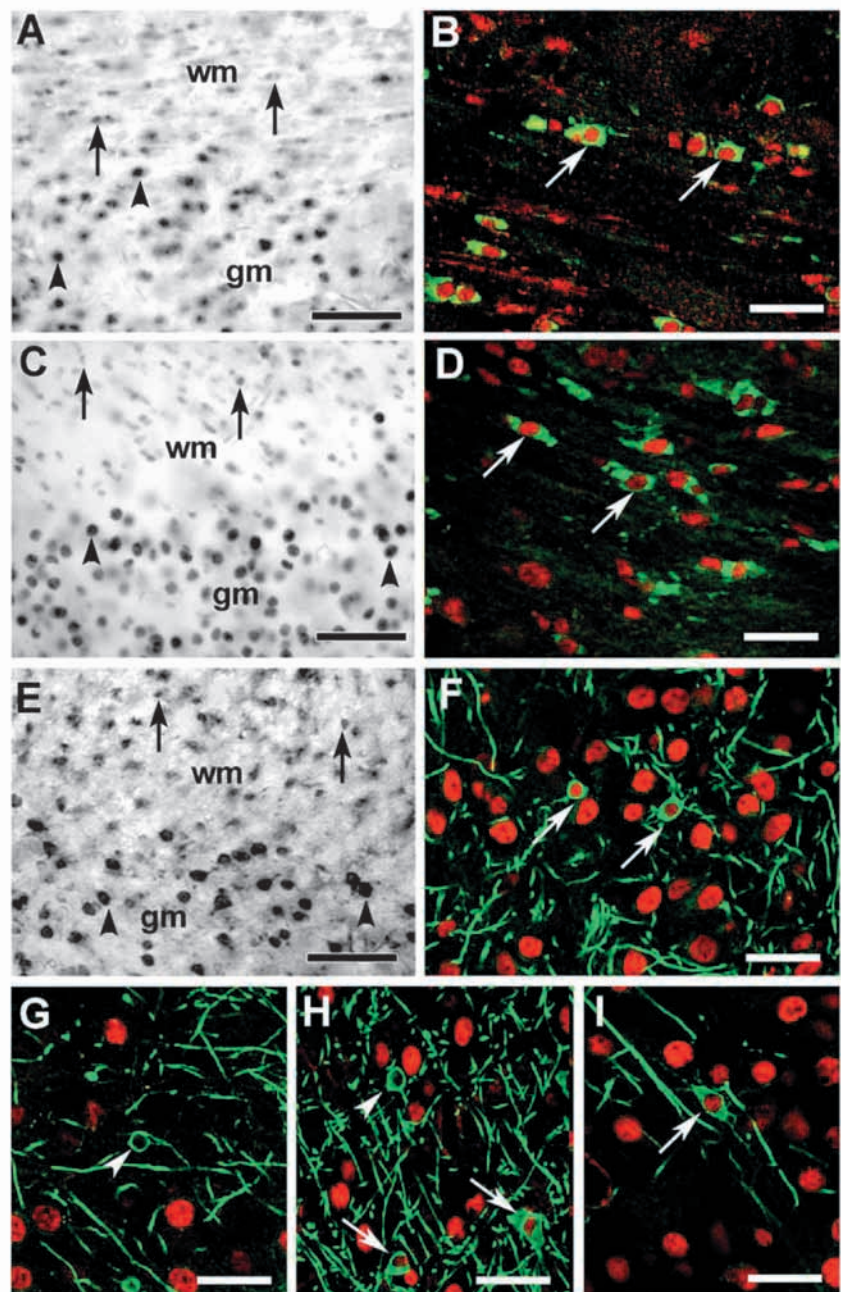
transport particles observed in many different cell types (Fig. 6A,B). An analysis of the KSRP (arrowheads in B, red) and hnRNP A2 (arrows, green) fluorescence in each granule demonstrated that KSRP-immunoreactive proteins and hnRNP A2 were concentrated in separate granule populations. The relative levels of KSRP and hnRNP A2 fluorescence in each granule were calculated, and a histogram of these values showed that the majority of granules contained just KSRP [red,  $r/(r+g)=1$ ] or hnRNP A2 [green,  $g/(r+g)=0$ ] (Fig. 6C). Few granules appeared to contain both proteins. This suggests that there are transport complexes that transport zipcode-containing mRNAs and separate granules that transport A2RE-containing mRNAs.

## Discussion

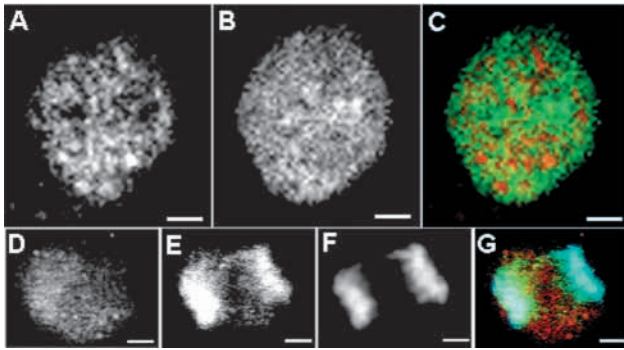
Understanding of the nature of RNA-protein interactions and the combinations of cis-acting sequences and trans-acting factors that govern many of the aspects of RNA metabolism in somatic cells has been limited by the paucity of well defined cis-acting sequences. By contrast, A2RE, zipcode and AURE are small oligonucleotides with defined binding properties and functions. We have shown that the sets of proteins that bind the  $\beta$ -actin zipcode and the AURE overlap extensively but do not include members of the A/B hnRNPs. Conversely, these A/B-type hnRNPs bind A2RE11 and A2RE but not the other two cis-acting sequences. Thus, most of the proteins we have identified are able to selectively bind more than one cis-acting element and each element binds several trans-acting factors. Vera/Vg1-binding protein and ZBP-1, which are almost identical proteins, provide further evidence for the same trans-acting factor recognizing various cis-acting elements: they operate on different cytoskeletal elements in various cell types and with different cis-acting elements (Oleynikov and Singer, 1998). Thus, if both proteins are involved in RNA trafficking, they

apparently operate through mutually exclusive sets of proteins.

The post-natal rat brain zipcode-binding proteins identified here include KSRP, FBP, hnRNP L, hnRNPs E1 and E2 and HuC. In recent experiments Gu and colleagues (Gu et al., 2001) identified ZBP-2, FBP, ssDBF (a single-stranded DNA binding protein) and ABBP (an hnRNP A/B type protein) as zipcode-binding proteins in embryonic chicken brain. There is overlap, with FBP and KSRP/ZBP-2 being common to both groups. ZBP-1 is the predominant zipcode-binding protein of embryonic fibroblasts (Ross et al., 1997), but expression of this protein declines after birth, and it was not detected in the zipcode-binding proteins from postnatal brain in our experiments. Little CRD-BP (a ZBP-1 homologue) was recently found in adult human brain using RT-PCR, although



**Fig. 4.** Zipcode- and A2RE-binding proteins are present in rat cortical neurons and glial cells. Sections of cortex of 15-day-old rats were stained for KSRP (A,B red), FBP (C,D red), hnRNP A2 (E,F red) and hnRNPs A1 (G red), B1 (H red), and A3 (I red) by immunoperoxidase (A,C,E) and immunofluorescence methods (B,D,F-I). Most neurons (arrows in A,C, E) and all white matter glia (arrowheads in A,C,E) were labeled for KSRP, FBP and hnRNP A2. Confocal microscopy of sections double-labeled with oligodendrocyte markers CC1 (green in B,D, which show regions of white matter) and CNP (green in F-I, which show cortical grey matter) detected KSRP, FBP, hnRNP A2 and hnRNP A3 in these cells (arrows). hnRNP A1 was not detected in oligodendrocytes (G, arrowhead), and many were also B1-negative (H, arrowhead). wm and gm: white and grey matter. Bars, 100  $\mu$ m for (A,C,E) and 25  $\mu$ m for other images.



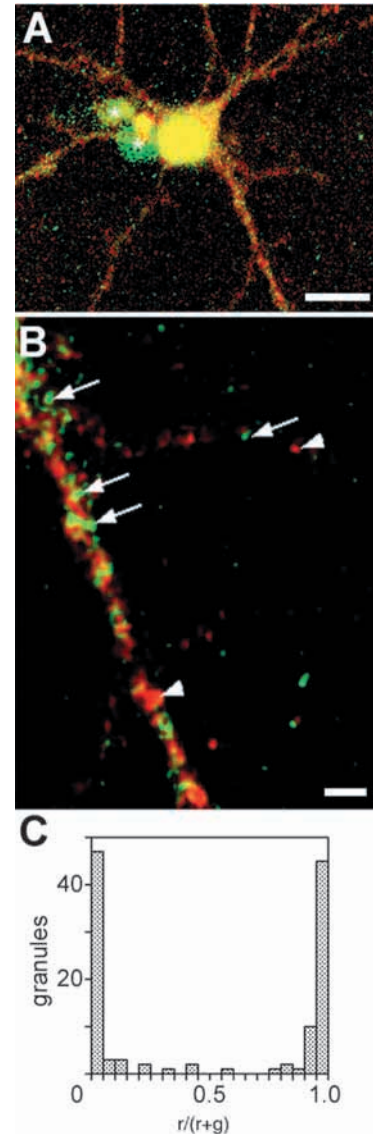
**Fig. 5.** KSRP and hnRNP A2 are concentrated in separate regions in the nuclei of cultured oligodendrocytes. KSRP (A and red in C; C2742 antibody) and hnRNP A2 (B and green in C; chicken antibody) are present at high levels in the nuclei with concentration of these proteins in regions that appear to contain primarily one protein or the other but not both. D-G show a mitotic cell in which KSRP (D, red in G) is concentrated in areas that largely exclude hnRNP A2 (E, green in G), which is associated with the dividing chromatin (F, blue DAPI staining in G). Bars, A-C, 2  $\mu$ m, D-G, 5  $\mu$ m.

it was detected in a few adult tissues, in many foetal tissues including the brain and in mesenchymal tumors (Ioannidis et al., 2001).

We saw only one closely spaced protein doublet in the molecular weight range expected for KSRP and ZBP-2: these proteins are most probably isoforms of KSRP, a rat homologue of the chicken ZBP-2 (Gu et al., 2001) and rat MARTA1 (Rehbein et al., 2000). The chicken ZBP-2 and human KSRP have over 86% identity at the amino-acid level but ZBP-2 has an additional segment near the N-terminus (Gu et al., 2001): searches of the human and mouse databases for this ZBP-2 47-residue segment did not reveal any cognate sequence.

The identification of several zipcode-binding proteins raises the question: do they interact with the zipcode-containing RNA in the nucleus, cytoplasm or in both locations? KSRP (Min et al., 1997) was reported to be located primarily in the nucleus, where it acts as a splicing factor and forms part of the perinucleolar structure (Huang, 2000), but our experiments show that it is also present in cytoplasmic granules that have been implicated in RNA trafficking (Hoek et al., 1998; Kiebler et al., 1999; Kohrmann et al., 1999) and binds zipcode, a known cis-acting sequence for cytoplasmic RNA trafficking.

Each of the other proteins has been implicated in the cytoplasmic metabolism of RNAs. FBP targets a far upstream cis-acting element of *c-myc*, regulating its transcription (Bazar et al., 1995), but it also binds a 26 nucleotide pyrimidine-rich sequence in the 3' UTR of GAP-43 mRNA, modulating its stability (Irwin et al., 1997; Wang et al., 1998). hnRNP E (Hahm et al., 1993) regulates cap- and IRES-dependent translation (Ostareck et al., 1997). It also binds and possibly stabilizes the pyrimidine-rich 3' UTR regions of erythropoietin (Czyzyk-Krzesk, 1999) and  $\alpha$ -globin (Kiledjian et al., 1995) mRNAs. hnRNP L (Piñol-Roma et al., 1989) was originally described as being localised to the nucleus (Huang, 2000; Kamma et al., 1995) but extranuclear functions are suggested by its association with gliomas that manifest translational repression and mRNA instability (Hamilton et al., 1999). It



**Fig. 6.** Extranuclear hnRNP A2 and KSRP are differentially localized. (A) A cultured oligodendrocyte showing the presence of KSRP (C2742 antibody, red) and hnRNP A2 (green) in the nucleus and processes. In the cell body the relative level of hnRNP A2 is higher. The two asterisks mark what appear to be adjacent cells. (B) A magnified view of a process of the cell from multiple merged optical sections. Distinct populations of granules containing KSRP (arrowheads) or hnRNP A2 (arrows) are evident in the oligodendrocyte processes. (C) A histogram showing the fluorescence ratios [red/(red + green); see Materials and Methods] of granules in four cells (excluding the cell shown in A) double-labeled for hnRNP A2 (green) and KSRP (red). Bars, A, 10  $\mu$ m, B, 2.5  $\mu$ m.

also binds the 126 nucleotide hypoxia stability region of human vascular endothelial growth factor mRNA (Shih and Claffey, 1999) and the AU-rich cis-acting destabilization sequence of glucose transporter Glut1 mRNA (Hamilton et al., 1999). Finally, HuC belongs to the ELAV family of RRM-containing proteins which, although found in the nucleus, binds AURE (Abe et al., 1996) and is colocalized with ribosomes in microtubule-associated granules in neuronal dendrites (Antic and Keene, 1998; Gao and Keene, 1996). FLAG-tagged HuC



is localized to the cytoplasm of transfected PC-12 cells (Akamatsu et al., 1999).

Thus, there is evidence to suggest that each of these zipcode- and AURE-binding proteins can bind to, and modulate the function of, cytoplasmic RNA, primarily by controlling mRNA stability or translation. This group includes proteins that contain KH domains, and some with RRM. Hence, the observed RNA binding cannot be attributed to a single type of RNA-binding domain: modules with divergent tertiary structures bind the  $\beta$ -actin zipcode sequence selectively.

The binding of these proteins to the zipcode and AURE can be partly rationalised in terms of their RNA-binding preferences. None of the RNA sequences that have been reported to bind to these proteins overlaps with A2RE11, but several have segments that match parts of the zipcode or AURE (Table 1): (1) FBP binds a 26 nucleotide sequence (Irwin et al., 1997) that shares the sequence UAUUU with AURE; (2) hnRNP E binds repeated CCUCCC sequences (Holcik and Liebhaber, 1997) overlapping with the zipcode CCCU; (3) hnRNP L binds the 33 nucleotide Glut1 stability element (Hamilton et al., 1999), which contains both zipcode (GUUUUA and UUACUG) and AURE (UUUA and UUUUUA) segments. It also binds the 21 nucleotide bovine vascular endothelial growth factor 3' UTR sequence (Shih and Claffey, 1999), which has limited overlap with the zipcode (UACA, ACCC); (4) HuC binds AUUUA and related AU-rich sequences (Abe et al., 1996; Inoue et al., 2000) and hence overlaps with the UUUA segment of the zipcode and the AURE sequence AUUUA. Thus, the identified zipcode- and AURE-binding proteins appear to favour U-rich tracts, which have more common in AURE than with zipcode.

Are the RNA segments identified above, which are often only four to six nucleotides, sufficient to mediate specific protein binding? In the few RNA-protein complexes of known 3D structure, linear stretches of eight to nine nucleotides are sufficient to fill the binding sites, and the essential nucleotides may be smaller in number. An 11 nucleotide segment of the A2RE is sufficient for recognition and transport by the hnRNP A2 targeting pathway (Munro et al., 1999), and of these 11 nucleotides, substitution in only five non-contiguous positions results in a marked lowering of binding to hnRNP A2 (Munro et al., 1999) (Moran-Jones et al., personal communication). It is possible that the zipcode-binding proteins recognise different segments of the 51 nucleotide zipcode oligonucleotide or the 22 nucleotide AURE. The KH3 domain of Nova has been shown to have an absolute requirement for the tetranucleotide UCAY presented in the loop region of a hairpin structure, although the two base pairs proximal to the loop also contribute to binding (Jensen et al., 2000).

In summary, we have shown that two cis-acting elements, zipcode and AURE, sequence selectively bind a similar group of proteins, six of which have recognized RNA-binding motifs. The A2RE11 also binds several proteins but they do not overlap with the zipcode/AURE-binding proteins. Although the zipcode-binding protein KSRP and the A2RE-binding hnRNP A2 are abundant in the nuclei of many CNS cells, they appear not to be colocalized. Similarly, both are present at lower levels in the processes of oligodendrocytes, but they are concentrated in mutually exclusive sets of putative transport granules. These experiments reinforce the view that binding of trans-acting factors to small cis-acting elements is sequence

selective but also demonstrate the possibility of competition between proteins for the same RNA element and, conversely, between different cis-acting elements for a single protein. The regulation of RNA metabolism is thus likely to involve a complex interplay between multiple RNA elements and trans-acting factors.

### Note added in proof

Rehbein et al. have recently shown that rat MARTA1 is the orthologue of human KSRP, with 98% amino acid identity.

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